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A Modified Plating Technique for the Recovery and Enumeration of Stressed *Salmonella typhimurium* Hf[1]

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ABSTRACT

A plating technique that would allow for recovery and enumeration of stressed *Salmonella typhimurium* was developed. The strain of *S. typhimurium* used in this study was isolated from an outbreak of salmonellosis caused by the consumption of contaminated pasteurized milk. Cultures propagated for 18 h at 37°C in Tryptic Soy Broth (TSB) were enumerated on Brilliant Green Agar (BGA), *Salmonella*-Shigella agar (SS), Xylose Lysine Desoxycholate Agar (XLD), and Hektoen Enteric Agar (HE). Recovery of 10^1 to 10^2 fewer cells was seen on the selective agars as compared to recovery on Tryptic Soy Agar (TSA). When the culture was spread plated onto TSA, allowed to stand at room temperature for 4 h, overlaid with XLD, HE, or SS, and incubated at 37°C for 24 h, recovery levels were comparable to recovery on TSA. Enhanced recovery of *S. typhimurium* cells that had been injured by freezing in TSB or in blended whole egg, or by heating at 54°C was also seen. In some cases recovery was at a higher level when the overlay procedure was used than on control TSA plates. Recovery of *S. typhimurium* from cheese that had been artificially contaminated with *S. typhimurium, Staphylococcus aureus,* and *Pseudomonas fragi* exceed control MPN values when the overlay procedure was used, although the recovery rate was within the MPN 95% confidence limits. The selective and differential properties of XLD, HE, and SS were maintained. *Salmonella* colonies appeared black, while the starter culture bacteria, *S. aureus* and *P. fragi* appeared as pinpoint white colonies. The potential exists for this procedure to be used for the direct enumeration of sublethally stressed salmonellae if their numbers exceed 100 per gram.

Bacterial injury caused by sublethal stress has been described as the inability of microorganisms to grow under conditions which are usually conducive to growth (*2,13*). It has been shown that bacterial injury can be caused by heating (*3*), freezing (*4,5,7,11*), freeze-drying (*9,10*), exposure to sanitizers (*13*), and other treatments encountered in food processing (*2*). Quantitative determination of bacterial injury has often been performed by comparing recovery of a sublethally stressed population on a nutritionally complete medium to recovery on a nutritionally minimal or selective medium. The number of injured cells would be the difference between the two counts (*3,4,5,6,7,8,9,10,11,13,15*). However, preliminary work in our laboratory showed that use of Brilliant Green Agar (BGA), Salmonella-Shigella Agar (SS), Xylose Lysine Desoxycholate Agar (XLD), and Hektoen Enteric Agar (HE) resulted in reduced recovery of *S. typhimurium* that had not been subjected to sublethal stress when compared to recovery on Tryptic Soy Agar (TSA). Reduced recovery of uninjured salmonellae on selective agars has been reported previously (*3,4,6*).

A plating procedure that allows for the detection of injured coliforms in foods has been described (*12*). The sample was initially plated on a nutritionally complete medium, followed by a 1 h incubation at room temperature, after which the plate was overlaid with Violet Red Bile Agar. A similar method is recommended for the detection of injured coliforms in dairy products (*1*). The objective of this research was to determine if this procedure could be modified to allow for more complete recovery of *Salmonella* on selective and differential agars.

MATERIALS AND METHODS

Preparation of inoculum

The strain of *S. typhimurium* used in this study was isolated from an outbreak of salmonellosis caused by the consumption of contaminated pasteurized milk. The culture was propagated at 37°C for 18-24 h in Tryptic Soy Broth (TSB) before use.

Recovery of unstressed S. typhimurium

Serial dilutions of the 18-24 h culture were prepared in 0.1% peptone water (PW). Triplicate 0.1 ml portions were spread onto the surface of pre-poured TSA, XLD, HE, SS, and BGA plates with sterile glass hockey sticks, and were incubated at 37°C for 24 h. Twelve additional TSA plates were inoculated and allowed to sit at room temperature (24°C) for 4 h. Twelve to 15 ml of XLD, HE, SS, or HE that had been tempered to 45°C was used to overlay the TSA plates. Three plates were prepared with each selective media. When the overlay solidified, the plates were incubated at 37°C for 24 h. All media used were supplied by Difco (Detroit, MI). TSA plates were prepared the day before use and were stored at room temperature. The selective plate media and media for overlay were prepared from the same lot

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just prior to use. Recovery of unstressed cells was done in this manner at least 4 times.

Recovery of freeze-injured S. typhimurium

An 18 h *S. typhimurium* culture was serially diluted in 0.1% PW. One ml of the dilutions that would result in a final population between 10 and 1000 cells/ml was added to 9 ml of either TSB or whole liquid eggs. The cultures were frozen at -20°C. The frozen cultures were thawed at room temperature and plated on TSA, the selective agars, and onto TSA overlaid with the selectives as previously described. Incubation of all plates was at 37°C for 24 h.

Recovery of heat injured S. typhimurium

One hundred fifty ml of an overnight culture was centrifuged at 8000 rpm for 8 min. The pellet was washed in Butterfield's phosphate buffer (BPB) (*14*) and centrifuged again. The cells were then resuspended in 150 ml of BPB which was blended for 30 sec to ensure even distribution of the cells. Ten-ml portions were transferred to screw-capped culture tubes which were placed in a waterbath at 54°C. The *S. typhimurium* population was determined at 0, 10, 20, and 30 min by plating on TSA, the selective agars, and on TSA overlaid with the selective media.

Recovery of S. typhimurium *from foods*

Colby cheese was artificially contaminated during manufacture with *S. typhimurium, Staphylococcus aureus,* and *Pseudomonas fragi.* These organisms were added to pasteurized milk immediately prior to the cheesemaking process. Eleven grams of cheese were blended in PW. Serial dilutions were spread plated directly onto the selective media and onto TSA overlaid with the selectives. The same dilutions were used to inoculate TSB in order to determine the *S. typhimurium* population by 3 tube most probable number (MPN) analysis. TSB tubes showing growth after 24 h at 37°C were used to inoculate Selenite-cystine and Tetrathionate broths. The selective broths were streaked onto XLD, HE, SS, and BGA. MPN tubes were considered positive if colony morphology on any selective plate was typical for salmonellae. All MPN tubes and selective plates were incubated at 37°C for 24 h.

RESULTS AND DISCUSSION

Reduced recovery of uninjured *S. typhimurium* was seen when plated directly onto the selective agars (Fig. 1). Use of the overlay procedure gave recovery levels comparable to those on TSA when XLD, SS, and HE were the overlay media. The most pronounced effect was seen when recovery directly on SS was compared to recovery on TSA overlaid with SS. No colony-forming units were apparent on SS plates prepared from dilutions containing 10^4 more cells than control TSA plates. When SS was used as an overlay, recovery was higher than 90% of the control. Enhanced recovery was less pronounced when BGA was the overlay medium, however, more than twice as many colonies were recovered than when plated directly on BGA.

When *S. typhimurium* was stressed by freezing for 9 d in TSB, the same pattern of recovery was seen (Fig. 2). Lower numbers were evident when plated directly on the selective media, and recovery approached that on TSA when the overlay technique was used. Again, the effect was not as pronounced when BGA was used as the overlay. Selective recovery was most efficient on TSA overlaid with SS agar. When *S. typhimurium* was frozen in egg for 30 d, use of the overlay procedure resulted in recovery at levels equal to or higher than recovery on TSA (Fig. 3). It is possible that some component in the egg is responsible for the enhanced recovery.

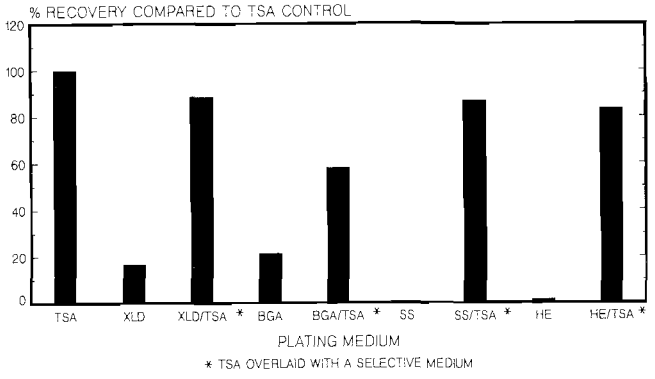

Figure 1. *Recovery of non-stressed* S. typhimurium.

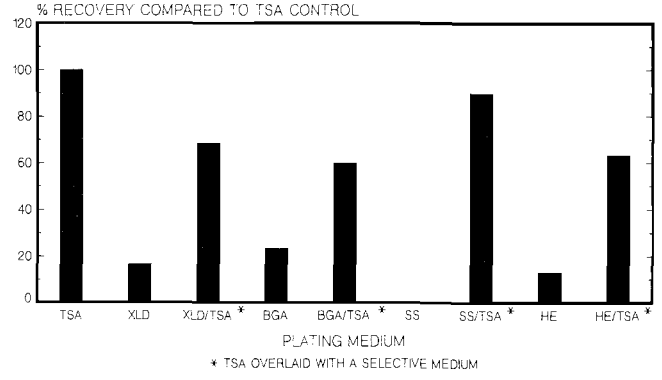

Figure 2. *Recovery of* S. typhimurium *stressed by freezing at -20°C in TSB.*

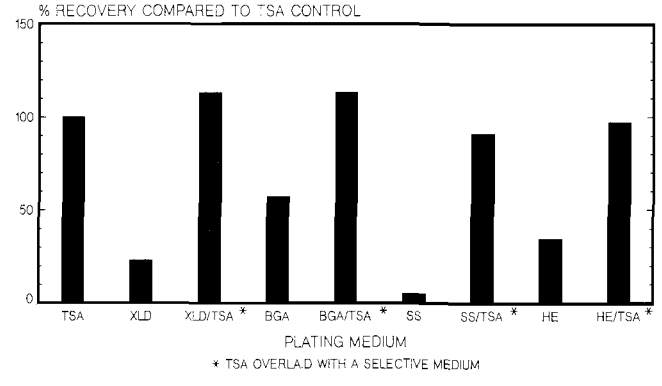

Figure 3. *Recovery of* S. typhimurium *stressed by freezing at -20°C in whole liquid egg.*

Heat-injury studies demonstrated reduced recovery of unstressed cells (heating time = 0 min) when *S. typhimurium* was plated on XLD or HE (Fig. 4). Plating on TSA overlaid with XLD resulted in higher recovery than on control plates. As heating time increased, percent recovery decreased on all types of plates; however, enhanced recovery was still apparent when the overlay technique was used as compared to recovery directly on the selective media.

The Colby cheese was found to have an MPN of 2.4 x 10^3 *S. typhimurium*/gram of cheese. The population determined direct plating onto the selective agars was lower than the MPN value (Fig. 5). The population determined by use of the overlay technique was equal to MPN value. Differential and selective properties of the media were retained when the overlay technique was used. *S. typhimurium* colonies appeared black, except on BGA where the colonies were pink-white as a result of the intense color of the surrounding medium. Lactic acid starter bacteria, *S. aureus*, and *P. fragi* appeared as pinpoint white colonies on plates prepared from the lowest dilutions used. Representative colonies of each morphology seen were used to inoculate Triple Sugar Iron Agar (Difco) and Lysine Iron Agar (Difco) slants to confirm that selective and differential properties were maintained. An advantage of the overlay procedure was that only 24 h were required to obtain isolated colonies on the selective media, while the MPN analysis required 3 d and the use of additional media.

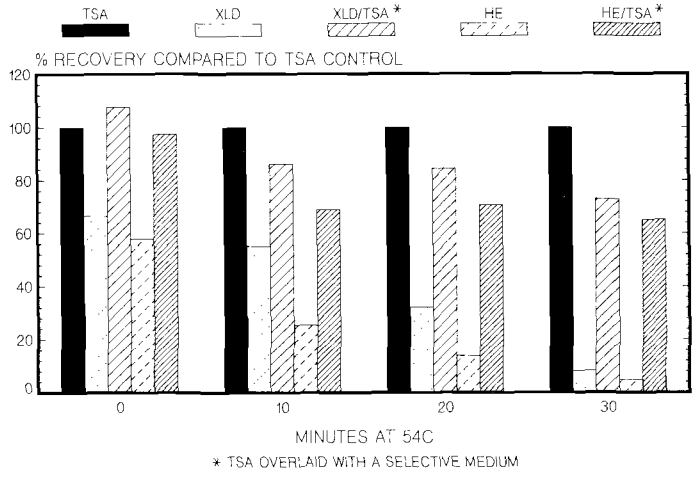

Figure 4. *Recovery of* S. typhimurium *stressed by heating at 54°C.*

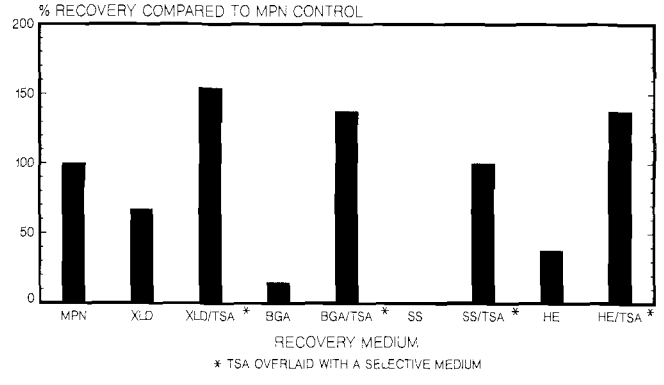

Figure 5. *Recovery of* S. typhimurium *from artificially contaminated Colby cheese.*

These results indicate that, when working with pure cultures, use of selective media for the enumeration of either unstressed or sublethally stressed *S. typhimurium* can result in recovery of fewer cells than when a selective medium such as TSA is used. Use of the overlay technique resulted in enhanced recovery of both unstressed *S. typhimurium* cells and cells stressed by freezing, heating, and the presence of acid and other microorganisms. The overlay media retained their selective and differential characteristics when the overlay procedure was used. This procedure was simple to perform and required no special equipment. It required less time and less media for the enumeration of salmonellae than the MPN procedure commonly used. The potential exists for this procedure to be used for the direct enumeration of salmonellae from food products that contain 100 or more salmonellae per gram.

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